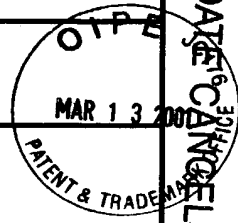
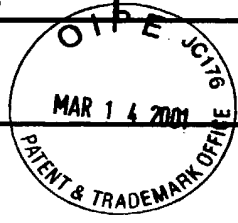


3-15-99 1011 Rec'd PCT/PTO 14 MAR 2001

FORM PTO-1390 (Modified) (REV 11-98)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER <b>6056-279</b>
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371			U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR <b>09/787216</b>
INTERNATIONAL APPLICATION NO. <b>PCT/US99/22756</b>	INTERNATIONAL FILING DATE <b>September 30, 1999</b>	PRIORITY DATE CLAIMED <b>October 1, 1998</b>	
TITLE OF INVENTION <b>Polymeric Hemoglobin Mutants</b>			
APPLICANT(S) FOR DO/EO/US <b>Clara Fronticelli</b>			



MAIL DATE 16 CANCELLED

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

- 1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
- 2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
- 3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
- 4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
- 5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
  - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☐ has been transmitted by the International Bureau.
  - c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US).
- 6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
- 7. ☒ A copy of the International Search Report (PCT/ISA/210).
- 8. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
  - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☐ have been transmitted by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☒ have not been made and will not be made.
- 9. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
- 10. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
- 11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
- 12. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

**Items 13 to 20 below concern document(s) or information included:**

- 13. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
- 14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
- 15. ☒ A **FIRST** preliminary amendment.
- 16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
- 17. ☐ A substitute specification.
- 18. ☐ A change of power of attorney and/or address letter.
- 19. ☒ Certificate of Mailing by Express Mail
- 20. ☒ Other items or information:

Express Mail Label No. EL 782181460 US  
Acknowledgement Postcard  
1 Diskette containing Sequence Listing; Operating System: Patent-In 2.0  
Statement Pursuant to 37 CFR 1.821(f)  
Applicant claims entitlement to small entity status.

**23973**  
PATENT TRADEMARK OFFICE

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR <div style="font-size: 2em; font-weight: bold;">09/787216</div>		INTERNATIONAL APPLICATION NO. <div style="font-weight: bold;">PCT/US99/22756</div>		ATTORNEY'S DOCKET NUMBER <div style="font-weight: bold;">6056-279</div>	
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21. The following fees are submitted: <b>BASIC NATIONAL FEE ( 37 CFR 1.492 (a) (1) - (5)) :</b>				CALCULATIONS    PTO USE ONLY	
<input type="checkbox"/> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO ..... <span style="float: right;">\$970.00</span>					
<input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO ..... <span style="float: right;">\$840.00</span>					
<input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... <span style="float: right;">\$690.00</span>					
<input type="checkbox"/> International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) ..... <span style="float: right;">\$670.00</span>					
<input checked="" type="checkbox"/> International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) ..... <span style="float: right;">\$96.00</span>					
ENTER APPROPRIATE BASIC FEE AMOUNT =				\$100.00	
Surcharge of \$130.00 for furnishing the oath or declaration later than <span style="float: right;"><input type="checkbox"/> 20    <input type="checkbox"/> 30</span> months from the earliest claimed priority date (37 CFR 1.492 (e)).				\$0.00	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	36    - 20 =	16	x    \$18.00	\$288.00	
Independent claims	3    - 3 =	0	x    \$80.00	\$0.00	
Multiple Dependent Claims (check if applicable). <span style="float: right;"><input checked="" type="checkbox"/></span>				\$260.00	
TOTAL OF ABOVE CALCULATIONS =				\$648.00	
Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) (check if applicable). <span style="float: right;"><input checked="" type="checkbox"/></span>				\$324.00	
SUBTOTAL =				\$324.00	
Processing fee of \$130.00 for furnishing the English translation later than <span style="float: right;"><input type="checkbox"/> 20    <input type="checkbox"/> 30</span> months from the earliest claimed priority date (37 CFR 1.492 (f)). <span style="float: right;">+</span>				\$0.00	
TOTAL NATIONAL FEE =				\$324.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable). <span style="float: right;"><input type="checkbox"/></span>				\$0.00	
TOTAL FEES ENCLOSED =				\$324.00	
				Amount to be: refunded	\$
				charged	\$

☐ A check in the amount of \_\_\_\_\_ to cover the above fees is enclosed.  
  
☒ Please charge my Deposit Account No. **19-1135** in the amount of **\$324.00** to cover the above fees.  
 A duplicate copy of this sheet is enclosed.  
  
☒ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. **19-1135** A duplicate copy of this sheet is enclosed.

**NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.**

SEND ALL CORRESPONDENCE TO:

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SIGNATURE  
 Daniel A. Monaco  
 NAME  
 30,480  
 REGISTRATION NUMBER  
 3-14-01  
 DATE

09/787216

JC02 Rec'd PCT/PTO 1 4 MAR 2001

**PATENT**

**Attorney Docket No.: 6056-279 US**

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re:	Patent application of	:International Application No.
	Clara Fronticelli	:PCT/US99/22756
		:
		:International Filing Date:
		:September 30, 1999
		:
Serial No.:	Not Yet Assigned	: Group Art Unit:
		: Not Yet Assigned
		:
Filed:	March 14, 2001	: Examiner:
		: Not yet assigned
		:
For:	<b>Polymeric Hemoglobin Mutants</b>	:

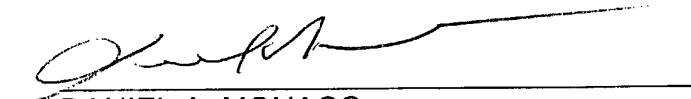
**STATEMENT PURSUANT TO 37 C.F.R. 1.821(f)**

Assistant Commissioner for Patents  
Box Patent Application  
Washington, DC 20231

Sir:

The information recorded in the computer readable form of the Sequence Listing is identical to the information in the written Sequence Listing.

Date: March 14, 2001

  
DANIEL A. MONACO  
Registration No. 30,480

09/787216  
JC02 Rec'd PCT/PTO 1 4 MAR 2001

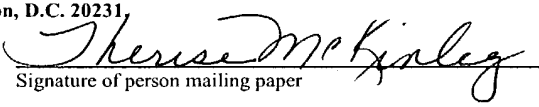
**PATENT**  
Attorney Docket No.: 6056-279

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE  
ACTING AS THE DESIGNATED/ELECTED OFFICE

In re: Patent application of Clara Fronticelli	:	International Application No.: PCT/US99/22756
U.S. Serial No.: Not Yet Assigned	:	International Filing Date: September 30, 1999
Filed: Concurrently Herewith	:	Group Art Unit: Not Yet Assigned
For: <b>Polymeric Hemoglobin Mutants</b>	:	Examiner: Not Yet Assigned

**PRELIMINARY AMENDMENT**

Kindly amend the above-identified patent application, without prejudice, as follows.

<b>CERTIFICATE OF MAILING UNDER 37 C.F.R. 1.10</b>	
EXPRESS MAIL Mailing Label Number: <u>EL 782181460 US</u>	
Date of Deposit: <u>March 14, 2001</u>	
I hereby certify that this correspondence, along with any paper referred to as being attached or enclosed, and/or fee, is being deposited with the United States Postal Service, "EXPRESS MAIL - POST OFFICE TO ADDRESSEE" service under 37 C.F.R. 1.10, on the date indicated above, and addressed to: Commissioner for Patents, Washington, D.C. 20231.	
	Signature of person mailing paper
<u>Therese McKinley</u>	Type or print name of person

**In the Specification:**

Page 1, cancel the paragraph entitled "Cross-Reference to Related Application" (inclusive of the section title).

Insert the following new paragraph immediately after line 1:

This is a 371 of PCT/US99/22756, international filing date September 30, 1999, which claims the benefit of U.S. provisional patent application Ser. No. 60/102,640, filed October 1, 1998. The entire disclosure of the aforesaid provisional application is incorporated herein by reference.

**In the Claims:**

Cancel claims 11 and 12.

Rewrite claims 5-7, 14, 15, 17 and 24 as follows. A mark-up of the original claims, showing the changes, is contained in Appendix A hereto.

5. (*amended*) A nucleotide sequence encoding a mutant polypeptide according to claim 4.

6. (*amended*) A nucleotide sequence according to claim 5 which comprises the nucleotide sequence SEQ ID NO:2.

7. (*amended*) A vector comprising a promoter operably linked to a nucleic acid sequence according to claim 5, capable of directing the expression of a mutant human  $\beta$ -globin polypeptide.

14. (*amended*) A modified human hemoglobin according to claim 10 or 13 comprising a mutant human  $\alpha$ -globin polypeptide comprising the amino acid sequence of normal human  $\alpha$ -globin modified by the substitution or deletion of Cys at position 104.

15. (*amended*) A modified human hemoglobin according to claim 14 wherein the mutant human  $\alpha$ -globin polypeptide is further modified by either the substitution of Cys for Ala at position 71, or the substitution of Cys for Ala at position 53.

17. (*amended*) A polymeric hemoglobin comprising a modified human hemoglobin according to claim 10, 13 or 16, wherein adjacent hemoglobins are covalently bonded to each other by one or more disulfide bridges formed by cysteine amino acid residues.

24. (*amended*) A mutant human  $\alpha$ -globin polypeptide according to claim 23, further modified by either the substitution of Cys for Ala at position 71, or the substitution of Cys for Ala at position 53.

- 4 -

**Remarks**

Claims 1-10 and 13-24 are pending in the application. Minor changes of an editorial nature have been made in the claims.

Respectfully submitted,

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Attorney for Applicant

Appendix A

- 5 -

5. (*amended*) A [nucleic acid] nucleotide sequence encoding a mutant polypeptide according to claim 4.

6. (*amended*) A [nucleic acid] nucleotide sequence according to claim [4] 5 which comprises the nucleotide sequence SEQ ID NO:2.

7. (*amended*) A vector comprising a promoter operably linked to a nucleic acid sequence according to claim 5 [6], capable of directing the expression of a mutant human  $\beta$ -globin polypeptide.

14. (*amended*) A modified human hemoglobin according to [any of claims 10-13] claim 10 or 13 comprising a mutant human  $\alpha$ -globin polypeptide comprising the amino acid sequence of normal human  $\alpha$ -globin modified by the substitution or deletion of Cys at position 104.

15. (*amended*) A modified human hemoglobin according to claim 14 [13 comprising a] wherein the mutant human  $\alpha$ -globin polypeptide [comprising the amino acid sequence of normal human  $\alpha$ -globin modified by the substitution or deletion of Cys at position 104, and] is further modified by either the substitution of Cys for Ala at position 71, or the substitution of Cys for Ala at position 53.

17. (*amended*) A polymeric hemoglobin comprising a modified human hemoglobin according to claim 10, [11, 12,] 13 or 16, wherein adjacent hemoglobins are covalently bonded to each other by one or more disulfide bridges formed by cysteine amino acid residues.

24. (*amended*) A mutant human  $\alpha$ -globin polypeptide [comprising the amino acid sequence of normal human  $\alpha$ -globin modified by the substitution or deletion of Cys at



Appendix A

- 6 -

position 104, and] according to claim 23, further modified by either the substitution of Cys for Ala at position 71, or the substitution of Cys for Ala at position 53.

**POLYMERIC HEMOGLOBIN MUTANTS****Reference to Government Grant**

The invention was supported in part by National Institutes of Health Grant HLBI P01-HL48517. The U.S. government has certain rights in the invention.

**Cross-Reference to Related Application**

The benefit of the filing date of U.S. provisional patent application Ser. No. 60/102,640, filed October 1, 1998, is hereby claimed. The entire disclosure of the aforesaid provisional application is incorporated herein by reference.

**Field of the Invention**

The invention relates to mutants of tetrameric hemoglobin molecules engineered to possess optimal oxygen carrying characteristics. The molecules are useful as blood substitutes.

**Background of the Invention**

Hemoglobin is a tetrameric molecule having a molecular weight of 64,500 Daltons. It is the protein in red blood cells which transports oxygen from the lungs to the tissues. The tetrameric molecule is formed of two pairs of  $\alpha$  and  $\beta$  subunits. The subunits are held together as a result of

ionic, hydrophobic and Van der Waals forces, and not as a result of covalent bonds. When hemoglobin is oxygenated, i.e., combined with oxygen, it readily forms  $\alpha$ - $\beta$  dimers having a molecular weight of 32,250 Daltons. These dimers are not retained *in vivo* by the kidneys and  
5 are eliminated through the urine. Urine elimination is the result of the dissociation of tetrameric hemoglobins into dimers.

Hemoglobin-based oxygen carriers have been proposed as substitutes for red blood cells, as artificial oxygen-delivery agents for replacing blood transfusions. Acellular products are preferred over cell-  
10 based red blood cell substitutes since cell-free products do not require tissue typing, are easier to store than cellular products, have a longer shelf life than cellular products, and can be made virus-free. However, there are problems associated with the use of hemoglobin solutions. Hemoglobin solutions have a short retention time in circulation due to the dissociation  
15 of hemoglobin into dimers which rapidly filter through the kidney glomeruli and vasoconstriction due to depletion of nitric oxide in the wall of the vasculature, probably resulting from hemoglobin extravasation.

In human red cells, the interaction of 2,3-diphosphoglycerate (2,3-DPG) with hemoglobin regulates the efficient release of oxygen to the  
20 tissues. Inside red blood cells, 2,3-DPG combines with hemoglobin in order to decrease its oxygen affinity to a level compatible with oxygen transport. Solutions of hemoglobin thus have a high oxygen affinity due to the absence of the 2-3-DPG regulation present within red cells.

An acellular product must have a high retention time in the  
25 circulation in order to serve as a blood substitute. In order to increase the retention time, it is necessary to impede the dissociation of the tetrameric hemoglobin molecule into dimers. Several types of chemical modifications have been implemented which stabilize the tetrameric form of hemoglobin by introducing an intramolecular cross-link between either the  $\alpha$  or the  $\beta$ -  
30 subunits (Chatterjee *et al.*, *J. Biol. Chem.* 261:9929-9937, 1986; Bucci *et al.*, *Biochemistry* 5:3418-3425, 1996; Jones *et al.*, *J. Biol. Chem.* 271:675-680,

1996) High molecular weight polymers have also been obtained by intermolecular chemical crosslinking although these comprise a large distribution of molecules of different molecular weight (Gould *et al.*, *Critical Care Clinics* 8:293-312, 1992; Gould *et al.*, *World J. Surg.* 20:1200-7, 1996).

5 However, such chemically cross-linked polymers must then be subjected to a series of complex purification steps to remove adjuvants or surfactants associated with the cross-linking process.

In order to maintain the hydric balance, solutions of tetrameric hemoglobin can not be used at a concentration higher than 7%, about half  
10 the concentration of hemoglobin in blood. Polymerization of several hemoglobin molecules makes possible the use of higher hemoglobin concentration without exceeding the normal oncotic pressure. By "polymerization" as used herein with respect to hemoglobin is meant the formation of associations of two or more hemoglobin tetramers.  
15 Polymerization also hinders extravasation of tetrameric hemoglobin molecules into the interstitial space apparently decreasing the vasoactivity of these solutions (Doyle *et al.*, *J. Biol. Chem.* 274:2583-2591, 1999).

Polymerization of tetrameric hemoglobin as a means of impeding dissociation involves the creation of higher molecular weight polymers.  
20 However, chemically-induced polymerization results in a large distribution of molecules of different molecular weight. Such heterogenous polymers tend to have varying characteristics across a population, the properties being difficult to control. A homogenous polymer is preferable.

The development of genetic engineering has made available the  
25 expression of hemoglobins in microorganisms. Certain mutant forms of hemoglobin have been discussed in the prior art. Hoffman *et al.* (US Patent Nos. 5,661,124 and 5,028,588) describe beneficial mutants having a  $P_{50}$  analogous to that of normal hemoglobin. The mutants are characterized by an osmolarity of greater than 303 mmol/L. Bonaventura  
30 & Riggs, *Science*, 158:800-2 (1967) describe the naturally occurring  $\beta$ -chain mutation "HbPorto Allegre" in which serine at position 9 is replaced

with cysteine.

A recombinant mutant hemoglobin currently under clinical trials has a low oxygen affinity and the tetrameric structure stabilized by using an expression vector containing a single duplicated tandemly fused  $\alpha$ -globin gene (Looker *et al.*, *Nature* 356:258-260, 1992). Another mutant recombinant hemoglobin contains five amino acid substitutions and has an intrinsic low oxygen affinity that is modulated by chloride ions (Fronticelli *et al.*, *J. Biol. Chem.* 270:30588-30592, 1995). The oxygen affinity values for this mutant are similar to those of whole blood in the presence of physiological chloride ions concentrations (120mM).

#### **Summary of the Invention**

It is an object of the invention to provide a mutant hemoglobin by recombinant DNA techniques having a high oxygen carrying capacity but low oncotic pressure useful as a blood substitute.

It is an object of the invention to provide a mutant hemoglobin which forms polymers of such hemoglobins through disulfide bonds in a controlled fashion, without interference from spurious bonds during refolding and polymerization.

It is a further object of the invention to provide a preparation of stable homogeneous polymers.

It is an object of the invention to provide for the formation of stable hemoglobin polymers without the use of exogenous synthetic chemical crosslinkers.

These and other objects of the invention will be apparent from the following disclosure.

According to the present invention, a modified human  $\beta$ -globin polypeptide is provided having the amino acid sequence of the normal human  $\beta$ -globin modified by (i) the substitution or deletion of Cys at positions 93 and 112, and (ii) the substitution of a Cys for a non-Cys amino acid at one other position. In preferred embodiments, Cys is substituted for

one of the amino acids at the following positions: (a) Ser at position 9; (b) Asn at position 80; or (c) Lys at position 17. Most preferably, Cys is substituted for Ser at position 9. According to a preferred embodiment, the cysteine residue at position 93 is substituted with an alanine residue, and/or the cysteine residue at position 112 is substituted with a glycine residue. Where all three substitutions are present (Ser9→Cys, Cys93→Ala and Cys112→Gly), the polypeptide has the sequence SEQ ID NO:4. A hemoglobin comprising the aforesaid three-position mutant  $\beta$ -globin and normal  $\alpha$ -globin is hereinafter identified as "Hb Prisca". Normal hemoglobin is referred to as "HbA". Normal hemoglobin is characterized by  $\alpha$ - and  $\beta$ -globin polypeptides having the native, i.e., non-mutant or wild type, amino acid sequences.

In another aspect, the invention is directed to nucleic acid encoding the aforesaid mutant  $\beta$ -globin polypeptide. In preferred embodiments, the nucleic acid encodes the polypeptide of SEQ ID NO:4. In one such embodiment, the nucleic acid has the nucleotide sequence SEQ ID NO:2.

In another aspect, the invention is directed to vectors comprising a promoter operably linked to a nucleic acid sequence encoding the mutant  $\beta$ -globin polypeptide, capable of directing the expression of a mutant human  $\beta$ -globin polypeptide. The invention is further directed to host cells transformed with such vectors.

A method for producing a human  $\beta$ -globin mutant polypeptide is provided comprising a culture of the aforesaid transformed host cells under conditions conducive to the expression of said polypeptide by said host cells.

The invention is also directed to a modified human hemoglobin in which at least one of the  $\beta$ -globins, preferably both  $\beta$ -globins, is a modified  $\beta$ -globin as described above. By "hemoglobin" is meant the functional tetrameric molecule comprising two  $\alpha$ -globin polypeptides, two  $\beta$ -globin polypeptides and heme. In one embodiment, the modified human hemoglobin is further characterized by the presence of an  $\alpha$ -globin mutant

polypeptide having the amino acid sequence of normal human  $\alpha$ -globin modified by the substitution or deletion of Cys at position 104. A preferred mutation is Cys104→Ser (SEQ ID NO:6). The  $\alpha$ -globin may be further modified by either the substitution of Cys for Ala at position 71, or the substitution of Cys for Ala at position 53. Both  $\alpha$ -globin polypeptides of the tetrameric hemoglobin are preferably mutant.

According to one embodiment, the modified hemoglobin has all of the native Cys residues substituted by non-Cys residues, and one non-native Cys is introduced into either the  $\alpha$ -globin or  $\beta$ -globin polypeptide, by substitution. Thus, a modified human hemoglobin comprises a mutant human  $\alpha$ -globin polypeptide comprising the amino acid sequence of normal human  $\alpha$ -globin modified by the substitution of Cys at position 104 by a non-Cys amino acid; and a mutant human  $\beta$ -globin polypeptide comprising the amino acid sequence of normal human  $\beta$ -globin modified by the substitution of Cys at positions 93 and 112 by non-Cys amino acids. The modified hemoglobin is further characterized by the substitution of Cys for the native sequence amino acid at one of the following positions:  $\beta$ -globin position 9;  $\beta$ -globin position 17;  $\beta$ -globin position 80;  $\alpha$ -globin position 71; or  $\alpha$ -globin position 53. Thus, since each hemoglobin contains two  $\alpha$  and two  $\beta$  chains, each molecule of modified human hemoglobin contains two non-native Cys residues.

The invention is further directed to a polymeric hemoglobin comprising the aforesaid modified human hemoglobins. The polymer may contain, for example, from 2 to 200 hemoglobin molecules, more preferably from 2 to 40, most preferably from 2-20. In one embodiment, the modified tetrameric hemoglobins of the polymeric hemoglobin are covalently bonded to each other by one or more intermolecular disulfide bridges formed by cysteine amino acid residues. In one embodiment, the modified hemoglobin of the polymer HB Prisca, and the polymer comprises from two to seven of the modified hemoglobins

The invention is also directed to an artificial blood substitute

comprising the aforesaid polymeric hemoglobin, preferably in combination with a pharmaceutically acceptable carrier. A method of supplementing the oxygen-carrying capacity of a patient's blood comprises administering to the patient an effective amount of the aforesaid blood substitute.

## 5 Brief Description of the Figures

Fig. 1 shows the normalized autocorrelation function (ACF) for normal hemoglobin HbA (•) and Hb Prisca (■). Solid lines show the non linear cumulant analysis fit. Fig. 2 shows the residual of the analysis for HbA and Hb Prisca ("Polymer").

10 Fig. 3 shows the ACF for HbA (•) and Hb Prisca (■) transformed according to the cumulant analysis method. Solid lines are a second order polynomial fit of the transformed data.

Fig. 4 shows oxygen binding curves of HbA (•) and of the heptad of Hb Prisca (■) at 110 days polymerization. The inset in Fig. 4, Fig. 5, 15 shows the cooperativity of these hemoglobins at the different levels of oxygen saturation.

## Detailed Description of the Invention

Novel purified hemoglobin mutants are provided comprising purified mutant  $\beta$ -globin polypeptides having the amino acid sequence of normal 20 human hemoglobin  $\beta$ -globin modified by (i) the substitution or deletion of Cys at positions 93 and 112, and (ii) the substitution of a Cys for a non-Cys amino acid at one other position. This mutation results in the polymerization of hemoglobin tetramers containing the mutant  $\beta$ -globin. The polymerization proceeds through the establishment of disulfide bonds. 25 The tetrameric hemoglobin molecules may be crosslinked to one another through cysteine residues, resulting in the formation of stable polymers that are highly purified. Preferably, Cys is substituted for Ser at position 9, Asn at position 80 or Lys at position 17. The Ser9→Cys substitution is preferred.



Unlike the naturally occurring HbPorto Allegre mutant which contains the same  $\beta$ (Ser9→Cys) substitution, the mutant  $\beta$ -globin of the present invention is further characterized by the deletion or substitution of cysteine residues at  $\beta$ -globin positions 93 and 112. This further modification prevents the formation of spurious S-S bonds during refolding and polymerization. According to a preferred embodiment, Cys93 is replaced by alanine and Cys112 is replaced by glycine, although other substitutions are possible. The resulting three-position mutation in the human hemoglobin  $\beta$ -globin,  $\beta$ (S9C+C93A+C112G) identifies "Hb Prisca".

Since the tetrameric hemoglobin molecule Hb Prisca contains two  $\beta$ -globin molecules and two  $\alpha$ -globin molecules, the three-position mutation results in a total of six mutations per tetramer. These mutations allow for proper folding of the recombinant product and increased yield. The mutant tetramers equilibrate to form a homogeneous hemoglobin polymer of seven individual hemoglobin tetramers. The heptamer has a molecular size near 450 kDa. (A single tetramer hemoglobin molecule has a size of 64 kDa)

Normal human  $\alpha$ -globin contains a Cys at position 104 (SEQ ID NO:5). According to the present invention, the  $\alpha$ -globin globins in the tetrameric hemoglobin molecule may contain a mutation whereby the Cys at position 104 is deleted or substituted with a non-cysteine amino acid. Optionally, a further modification may comprise insertion of a Cys into the  $\alpha$ -globin chain, or substitution of a Cys residue for another amino acid, at a position other than position 104. For example, Cys may be substituted for Ala at position 71, or Cys may be substituted for Ala at position 53. Preferably, the Cys at  $\alpha$ 104 is substituted with a non-Cys amino acid, most preferably Ser, and no other Cys residues are contained in the  $\alpha$ -globin polypeptide. Set forth as SEQ ID NO:7 and SEQ ID NO:6 are, respectively, the nucleotide and amino acid sequences of the Cys104→Ser  $\alpha$ -globin mutant.

According to another embodiment of the invention, the modified hemoglobin has all of the native Cys residues substituted by non-Cys

residues. To obtain polymerization, one non-native Cys is introduced into either the  $\alpha$ -globin or  $\beta$ -globin polypeptide, by substitution. The substitution for Cys occurs at  $\beta$ -globin Ser9;  $\beta$ -globin Lys17;  $\beta$ -globin Asn80;  $\alpha$ -globin Ala 71; or  $\alpha$ -globin Ala53. The resulting modified hemoglobin molecule  
5 contains two non-native Cys residues per molecule, since each hemoglobin molecule contains two  $\alpha$  and two  $\beta$  chains. Polymerization proceeds through the artificially introduced Cys residues.

The polymerization of hemoglobin tetramers containing the Hb Prisca mutation according to the present invention has been observed by  
10 light scattering measurements which estimate the radius of the molecules in solution. Dynamic light scattering indicates that the polymer is formed by seven hemoglobin tetramer molecules, and that the polymer is highly homogeneous. The functional characteristics are those of natural human hemoglobin.

15 Hb Prisca polymerizes to generate a homogeneous globular polymer of human hemoglobin with functional characteristics similar to those of the natural protein. Polymerization of hemoglobin tetramers containing the Hb Prisca mutation to about 5 molecules occurs in 6 days; polymerization to 7 molecules is reached after about 3 months. The oxygen affinity  $P_{50}$   
20 (partial pressure of oxygen corresponding to 50% saturation with oxygen) of the heptamer is the same as that of normal tetrameric human hemoglobin (HbA). Moreover, the cooperativity (n), another parameter relevant to oxygen delivering capacity, is maintained on polymerization. The cooperativity (n) is only slightly decreased with respect to native  
25 hemoglobin. The heptamer is resistant to autoxidation. The heptamer is highly stable with absence of precipitation and methemoglobin formation during various manipulations and upon storage for 120 days at 4°C.

Since oncotic pressure is proportional to the number of molecules in solution, solutions of the polymerized hemoglobin of the present  
30 invention are expected to have a low oncotic pressure per gram of protein, while maintaining a high oxygen carrying capacity.

A model was constructed of R-state Hb Prisca tetramers joined by disulfide bridges between their  $\beta$ Cys9 residues. The tetramers were arranged in the best approximation to the corners of a cube without van der Waals interpenetration. The model predicts a location for an eighth tetramer to complete the cube, if the entropic costs of disulfide coupling the eighth tetramer were not so high. This model shows that if the tetramers arranged themselves in the approximation of a cube, the remaining sulfhydryls would not be accessible to additional polymerization after the addition of the seventh molecule.

The amino acid sequences of the normal human  $\alpha$ -globin and  $\beta$ -globin polypeptides are known and set forth herein: SEQ ID NO:5 ( $\alpha$ -globin); SEQ ID NO:3 ( $\beta$ -globin). The nucleotide sequences encoding the normal polypeptides are also known and set forth herein: SEQ ID NO:12 ( $\alpha$ -globin); SEQ ID NO:1 ( $\beta$ -globin).

The mutant hemoglobin  $\beta$ -globins of the present invention may be prepared utilizing well-known expression systems for producing recombinant  $\beta$ -globin in suitable microbial hosts such as *E. coli*. See Fronticelli *et al.*, *J. Prot. Chem.* 10:495-501(1991), Sanna *et al.*, *J. Biol. Chem.* 272: 3478-3486 (1996) and U.S. Patent 5,239,061 to Fronticelli *et al.*, the entire disclosures of which are incorporated herein by reference. Site-specific mutagenesis is used to introduce appropriate mutations into the native  $\beta$ -globin nucleotide coding sequence to provide a mutant DNA encoding the desired mutant  $\beta$ -globin polypeptide.

Similarly, site-specific mutagenesis is used to introduce appropriate mutations into the native  $\alpha$ -globin nucleotide coding sequence to provide a mutant DNA encoding the desired mutant  $\alpha$ -globin polypeptide. Such  $\alpha$ -globin mutants may be prepared, for example, according to the procedures set forth in U.S. Patent 5,661,124 to Hoffman *et al.*, the entire disclosure of which is incorporated herein by reference. Mutants of  $\alpha$ -globin may also be prepared using the  $\alpha$ -globin expression plasmid, pNF $\alpha$ . The construction of pNF $\alpha$  is described by Sanna *et al.*, *J. Biol. Chem.* 272:3478-



encoding the mutant  $\alpha$ - and  $\beta$ -globins of the present invention. These modified sequences are used to produce mutant  $\alpha$ - and  $\beta$ -globins, and mutant hemoglobins containing such mutant globins.

DNA regions are operably linked when they are functionally related  
5 to each other. For example, DNA for a pre-sequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a pre-protein which participates in the secretion of the polypeptide; a promoter is operably linked to a coding sequence if it controls the transcription of the sequence; or a ribosome binding site is operably linked to a coding  
10 sequence if it is positioned so as to permit translation. Generally, "operably linked" means contiguous and, in the case of secretory leaders, contiguous and in reading phase.

Suitable host cells for expressing the mutant globins are prokaryotes, yeast or higher eukaryotic cells. Prokaryotes include gram  
15 negative or gram positive bacteria, for example *E. coli* or *Bacilli*. Higher eukaryotic cells include established cell lines of insect, spider or mammalian origin.

Generally, a microbial vector will contain an origin of replication recognized by the intended host, a promoter which will function in the host  
20 and a phenotypic selection gene, for example, a gene encoding proteins conferring antibiotic resistance or supplying an auxotrophic requirement.

Vectors must contain a promoter which is recognized by the host organism. This is generally a promoter homologous to the intended host. Promoters most commonly used in recombinant DNA construction include  
25 the  $\beta$ -lactamase (penicillinase) and lactose promoter systems, a tryptophan (*trp*) promoter system and the *tac* promoter. While these are the most commonly used, other known microbial promoters are suitable. Details concerning their nucleotide sequences have been published, enabling a skilled worker operably to ligate them to DNA encoding  $\alpha$ - and/or  $\beta$ -globins.

30 In addition to prokaryotes, eukaryotic microbes such as yeast cultures may be transformed with  $\alpha$ - and/or  $\beta$ -globin encoding vectors.

*Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms, although a number of other strains are commonly available. Yeast vectors generally will contain an origin of replication from the 2 micron yeast plasmid or an  
5 autonomously replicating sequence (ARS), a promoter, a DNA sequence coding for  $\alpha$ - and/or  $\beta$ -globins, sequences for polyadenylation and transcription termination and a selection gene.

Suitable promoting sequences in yeast vectors include the promoters for metallothionein, 3-phosphoglycerate kinase or other  
10 glycolytic enzymes. One such yeast vector is described by Wagenbach *et al.*, *BioTechnology* 9:57 (1991).

Other promoters, which have the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative  
15 enzymes associated with nitrogen metabolism, and the aforementioned metallothionein and glyceraldehyde-3-phosphate dehydrogenase, as well as enzymes responsible for maltose and galactose utilization. In constructing suitable expression plasmids, the termination sequences associated with these genes are also ligated into the expression vector 3'  
20 of the globin coding sequence to provide polyadenylation of the mRNA and termination.

In addition to microorganisms, cultures of cells derived from multicellular organisms may also be used as hosts. In principal, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate  
25 culture. However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years. Examples of useful host cell lines are VERO and HeLa cells, Chinese Hamster ovary (CHO) cell lines, and WI38, BHK, COS-7 and MDCK cell lines. Expression vectors for such cells  
30 ordinarily include (if necessary) an origin of replication, a promoter located upstream from the gene to be expressed, along with a ribosome binding

site, RNA splice site (if intron-containing genomic DNA is used), a polyadenylation site, and a transcriptional termination sequence.

The modified human hemoglobin of the present invention may also be produced in transgenic animals. Hemoglobins have been produced in transgenic pigs, for example. See U.S. Pat. 5,922,854 to Kumar *et al.*

The transcriptional and translational control sequences in expression vectors to be used in transforming vertebrate cells are often provided by viral sources. For example, commonly used promoters are derived from polyoma, Adenovirus 2, and most preferably Simian Virus 40 (SV40). The early and late promoters are particularly useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication. Smaller or larger SV40 fragments may also be used, provided the approximately 250 bp sequence extending from the Hind III site toward the BglI site located in the viral origin of replication is included.

An origin of replication may be provided either by construction of the vector to include an exogenous origin, such as may be derived from SV40 or other viral (e.g., Polyoma, Adenovirus, VSV, or BPV) source, or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter is often sufficient.

According to one embodiment of the invention, the  $\beta$ -globin and  $\alpha$ -globin polypeptides, including mutants thereof, are advantageously expressed as fusion proteins. The normal  $\beta$ -globin polypeptide may be expressed as the fusion protein NS1-FX- $\beta$ -globin. This fusion protein comprises 81 residues of the flu virus protein NS1, the Factor X<sub>a</sub> recognition sequence Ile-Glu-Gly-Arg (SEQ ID NO:8), and the sequence of  $\beta$ -globin. The fusion protein is expressed in *E. coli* AR58 by transformation with plasmid pJKO5, the construction of which is described by Fronticelli *et al.*, *J. Prot. Chem.* 10:495-501(1991), the entire disclosure of which is incorporated herein by reference. The mutant  $\beta$ -globin polypeptides are prepared in the same fashion, substituting the wild type  $\beta$ -globin nucleotide sequence in pJKO5 with the appropriate mutant sequence generated by

site-specific mutagenesis. A procedure for introducing mutations in the  $\beta$ -globin nucleotide sequence is described in Patent 5,239,061. Site-directed mutagenesis may be carried out using standard molecular biological techniques, using standard reagents such as the "Muta-Gene M-13 in vitro  
5 Mutagenesis Kit" available from Bio-Rad Laboratories.

According to one embodiment of the invention, the  $\beta$ -globin of Hb Prisca is prepared as follows. The 934 bp fragment from HindIII digestion of pJKO5 is isolated and cloned into the HindIII site of the M13mp18 phage vector, and mutagenesis is carried out with the Bio-Rad kit. The following  
10 oligonucleotides are used to introduce the indicated mutations into  $\beta$ -globin, wherein the mutating nucleotides are underlined:

$\beta$ (Ser9→Cys): 5'-GGC AGT AAC GGC GCA CTT CTC CTC AGG-3'  
(SEQ ID NO:9);

$\beta$ (Cys93→Ala): 5'-TGC AGC TTG TCA GCA TGC AGC TCA CTC-3'  
15 (SEQ ID NO:10); and

$\beta$ (Cys112→Gly): 5'-CAG CAC ACC GAC CAG CAC-3' (SEQ ID NO:11).

For expression, the mutated genes are removed from the M13mp18 plasmid with HindIII and ligated back into the 6200 bp fragment obtained  
20 from digestion of pJKO5 with HindIII.

Mutants of  $\alpha$ -globin are prepared using the  $\alpha$ -globin expression plasmid, pNF $\alpha$ , which is structurally analogous to pJKO5. The construction of pNF $\alpha$  is described by Sanna *et al.*, *J. Biol. Chem.* 272:3478-3486 (1997), the entire disclosure of which is incorporated herein by reference.  
25 Expression of the plasmid in *E. Coli* strain AR120 yields large amounts of the NS1-FX- $\alpha$ -globin fusion protein, which is analogous to the NS1-FX- $\beta$ -globin fusion protein. Mutagenesis is carried out with the appropriate mutagenizing oligonucleotides, as with pJKO5, to yield the desired  $\alpha$ -globin mutants.

30 The partially purified fusion proteins may be dissolved in alkali and partially renatured by extensive dialysis at pH 8 to 9 in the presence of an



S-S bond reducing agent. After enzymatic cleavage with factor Xa, the globin polypeptide separates. The isolated  $\beta$  and  $\alpha$  polypeptides may be combined and heme added to reconstitute tetrameric hemoglobin. It may be appreciated that the reconstituted hemoglobin may comprise mutant  $\beta$ -globin and native  $\alpha$ -globin, or both mutant  $\beta$ -globin and mutant  $\alpha$ -globin. To form a functional hemoglobin molecule, it is necessary to join the  $\alpha$ - and  $\beta$ -globins, and provide the prosthetic heme groups. Procedures for the purification of cloned mutant  $\beta$ -globin and reconstitution of tetrameric hemoglobin are disclosed in Patent 5,239,061.

Upon treatment of the tetrameric hemoglobin with *p*-chloromercuribenzoate (Bucci and Fronticelli, *J. Biol. Chem.* 240, PC551, 1965), the  $\alpha$  and  $\beta$  mutant chains can be separated in their native state. Mixing of the separated native state mutant  $\alpha$  and  $\beta$  chains gives a tetrameric hemoglobin carrying mutations in both  $\alpha$ - and  $\beta$ -globins.

In one embodiment, both the  $\beta$ -globin and  $\alpha$ -globin polypeptides are recombinantly produced. In such cases, it is desirable to co-express the polypeptides in the same expression system. One such co-expression system is described in U.S. Pat. No. 5,753,465 to Ho *et al.*, the entire disclosure of which is incorporated herein by reference. The expression method utilizes a plasmid, pHE2, in which the synthetic human  $\alpha$ - and  $\beta$ -globin genes and the Met-AP gene for *E. coli* are arranged in tandem in the same orientation and are co-expressed under the control of separate *tac* promoters. The coding sequence the *E. coli* Met-AP gene is contained in ATCC 53245 and U.S. Pat. No. 4,865,974, 4,870,017 and 5,013,662, the entire disclosures of which are incorporated herein by reference. The coding sequence is inserted into the EcoRI and HindIII sites of the HbA expression plasmid pDLIII-13e, the construction of which is disclosed by Hoffman *et al.*, *Proc. Natl. Acad. Sci. USA* 87:8521 (1993), the entire disclosure of which is incorporated herein by reference. The resulting plasmid, pTC-EM2, contains the complete coding sequence of the *E. coli* Met-AP gene flanked by the *tac* promoter and 5ST1T2 terminators from

pDLIII-13e.

The resulting co-expression plasmid contains two expression cassettes arranged in tandem in the same orientation as follows: (1) *tac* promoter-*E. coli* Met-AP coding sequence-5ST1T2 terminator and (2) *tac* promoter- $\alpha$ -globin coding sequence- $\beta$ -globin coding sequence-5ST1T2 terminator. See Figure 1 of the Ho *et al.* patent.

The modified hemoglobin polymers of the present invention may be incorporated into physiologically acceptable blood substitute solutions. One such solution may be based upon the solution described in U.S. Pat. 5,028,588 to Hoffmann *et al.* The solution contains from about 6 to about 120 g/L hemoglobin polymer, from about 135 to about 145 mEq/L sodium, from about 3.5 to about 4.5 mEq/L potassium, and from about 90 to about 100 mEq/L chloride. Preferably, the solution has a pH of 7.3-7.5, an osmolarity of 280-310, and an oncotic pressure of 20-30 mm Hg. Glucose may be optionally added to adjust the osmolarity. Additional agents such as albumin, dextran and polyethylene glycol, in the amount suggested in Pat. 5,028,588, may be added to increase oncotic pressure. Antioxidants and/or free radical scavengers such as mannitol, glutathione, ascorbic acid or vitamin E may also be included.

The blood substitute may be administered to a patient in any situation requiring supplementation of the oxygen-carrying capacity of the patient's blood. The amount of the blood substitute administered will depend on the size, weight and age of the patient, the clinical condition of the patient, and the degree of impairment of the natural oxygen-carrying capacity of the patient's blood. Herein, an "effective amount" of blood substitute is any amount which brings about a clinically relevant enhancement of the oxygen-carrying capacity of the patient's blood over a clinically meaningful time interval.

The practice of the invention is illustrated by the following non-limiting examples.

### **Example 1**

#### **Preparation of Mutant and Wild Type**

##### **$\beta$ -Globin Fusion Protein Vector**

The construction of the plasmid pJKO5 is described by Fronticelli *et al.*, *J. Prot. Chem.* 10:495-501(1991), the entire disclosure of which is incorporated herein by reference. The vector is a derivative of pAS1 (Rosenberg, *et al.* (1982) *Met. Enzymol.* 101, 123), where the NS1 gene from influenza A virus is cloned into the Bam HI site (Young, *et al.* (1983) *Proc. Natl. Acad. Sci USA* 80, 6105). A linker coding for the recognition sequence of blood clotting Factor X<sub>a</sub> is inserted into the NcoI site of the NS1 gene. The human  $\beta$ -globin cDNA (obtained by Dr. B. Forget, Yale University) is inserted 3' to the factor X<sub>a</sub> recognition sequence giving a plasmid similar to the described previously by Nagai and Thogersen (1984), *Nature* 308, 810. High expression levels of the NS1-FX- $\beta$ -globin fusion protein are obtained using *E. coli* AR58 transformed with this plasmid. The fusion protein comprises 81 residues of protein NS1, and the Factor Xa recognition sequence Ile-Glu-Gly-Arg (SEQ ID NO:8), and  $\beta$ -globin. The fusion protein is expressed in *E. coli* AR58 by transformation with plasmid pJKO5.

For mutagenesis, plasmid pJKO5 was subjected to HindIII digestion. The 934 bp digestion fragment was isolated and cloned into the HindIII site of the M13mp18 phage vector. Mutagenesis was carried out on the resulting plasmid using the Muta-Gene mutagenesis kit (BioRad) and the following oligonucleotides where mutating nucleotides are underlined:

$\beta$ (Ser9→Cys): 5'-GGC AGT AAC GGC GCA CTT CTC CTC AGG-3' (SEQ ID NO:9);

$\beta$ (Cys93→Ala): 5'-TGC AGC TTG TCA GCA TGC AGC TCA CTC-3' (SEQ ID NO:10); and

$\beta$ (Cys112→Gly): 5'-CAG CAC ACC GAC CAG CAC-3' (SEQ ID NO:11).

The mutated gene was removed from the M13mp18 plasmid with HindIII

and ligated back into the 6200 bp fragment obtained from digestion of pJKO5 with HindIII.

### **Example 2**

#### **Preparation of Mutant and Wild type $\alpha$ -Globin Fusion Protein Vector**

5            Vectors for expression of mutant  $\alpha$ -globin may be prepared by mutagenizing the  $\alpha$ -globin expression plasmid pNF $\alpha$ . The construction of the latter is described by Sanna *et al.*, *J. Biol. Chem.* 272:3478-3486 (1997), the entire disclosure of which is incorporated herein by reference. The  $\alpha$ -globin expression plasmid, pNF $\alpha$ , is structurally identical to pJKO5  
10           except that the  $\alpha$ -globin cDNA replaces the  $\beta$ -globin cDNA of pJKO5. For mutagenesis, plasmid pNF $\alpha$  is subjected to HindIII digestion and the 934 bp digestion fragment is isolated and cloned into the HindIII site of the M13mp18 phage vector, followed by mutagenesis with the appropriate oligonucleotides to introduce the desired change, e.g., the substitution of  
15           Cys104 to a non-cysteine amino acid, in the amino acid sequence of  $\alpha$ -globin.

### **Example 3**

#### **Expression, Reconstitution and Purification of Recombinant Hemoglobin Containing Mutant $\beta$ -Globin (Hb Prisca)**

20           Growth, expression and purification of recombinant  $\beta$ -globin, and reconstitution and assembly of recombinant  $\beta$ -globin with  $\alpha$ -globin into tetrameric hemoglobin, followed protocols previously described by Fronticelli *et al.*, *J. Prot. Chem.* 10:495-501(1991), Sanna *et al.*, *J. Biol. Chem.* 272: 3478-3486 (1996), with modifications. *E. coli* strain AR120  
25           was transformed with the mutant pJKO5 vector prepared above. Cells were grown in a fermentor at 37°C in an LB plus ampicillin media to an OD<sub>600</sub> of 0.600. Nalidixic acid was added (60  $\mu$ g/ml) and the growth continued for 4 hours. The cells were harvested either by centrifugation or using a Pellicon Cassette. The fusion protein was separated from most of

the other cell components by washes with deoxycholate and Triton X-100. The insoluble pellet collected by centrifugation contained the fusion  $\beta$ -globin protein along with some other residual proteins. The pellets were solubilized quickly in a minimum volume of 50 mM cold NaOH and diluted

5 10-fold with 40 mM borate buffer at pH 8.4. This sample was digested with Factor  $X_a$  in the ratio 1:200 in the presence of 1 mM  $Ca^{2+}$  for 24 hours. The extent of enzymatic cleavage was monitored by reverse phase HPLC using a Vydac C4 column. Upon cleavage with Factor  $X_a$ , the protein was diluted to 2 mg/ml ( $OD_{280} = 1.0$  is taken as 1 mg/ml) with 0.04 M borate buffer at pH

10 9.0 + 0.002 M EDTA. In order to dissolve the aggregates, the solution was deoxygenated by stirring under a constant stream of  $N_2$  followed by addition of dithiothreitol (DTT) to a final concentration of 0.001 M. After 24 hours at 4°C the protein was reconstituted with CN-heme and native partner  $\alpha$ -subunit prepared from carboxy HbA by reaction with p-

15 chloromercuribenzoate (Bucci and Fronticelli, *J. Biol. Chem.* 240, PC551, 1965). After one week the protein was concentrated and the heme converted to the carbonmonoxy derivative by passage of the sample on a Sephadex 25G column, equilibrated with 0.1 M Phosphate buffer pH 7.3 + 0.03mM DTT saturated with carbon monoxide (CO), to which 2 ml Na-

20 dithionite (100mg/ml) had been previously absorbed. The hemoglobin was purified on an affinity column of immobilized hemoglobin (Rossi Fanelli *et al.*, *Eur. J. Biochem.* 92:253-259:1978; Chiancone *et al.*, *J. Chromatogr.* 604:117-123, 1992). All the purification steps were performed in the presence of 0.03 mM DTT in order to avoid any S-S formation during the

25 purification procedure. After purification of the tetrameric mutant Hb, the DTT present in the solution was removed by passage through a G25 Sephadex column, equilibrated with a buffer containing 50 mM Hepes + 100mM NaCl+ 1mM EDTA saturated with CO. This step is referred to as the "zero polymerization time". The sample was concentrated in the cold

30 to 1.5-2.0 mM for 120 days.

#### **Example 4**

##### **Measurement of Dispersivity of Hb Prisca**

##### **Polymer by Dynamic Light Scattering**

The radius of Hb Prisca was measured as follows by dynamic light  
5 scattering. The radius was measured at time zero prior to the removal of  
DTT, while measurements at increasing time were recorded after removal  
of the DTT.

##### **A. Methods**

Dynamic light scattering measurements were performed using the  
10 instrument DynaPro-801. A Diode laser provides a light source at 783 nm.  
The diffused light was collected at a scattering angle of 90° through fiber  
optics and converted to an electrical signal by an avalanche photo diode.  
The scattering cell is a flow-through type and the inlet and outlet ports are  
designed such that the flow pattern would sweep the entire cell volume,  
15 allowing maintenance of a low number as possible the number of foreign  
particles in the scattering volume by carefully cleaning of the cell prior to  
the measurements. The hemoglobin in the carbonmonoxy form was at a  
concentration between 1 and 2 mg/ml in 50 mM Hepes +100 mM NaCl  
+1mM EDTA buffer at pH 7.4 equilibrated with CO. The samples were  
20 slowly injected into the scattering cell through a series of Whatman  
inorganic membrane filters with decreasing porosity from 0.1µm to 0.02µm.

The time-dependent autocorrelation function (ACF) of the photon  
current was then computed with a software correlator (based on a DSP  
unit) with 83 channels. Every 10 seconds an ACF was collected with  
25  $10^5$ - $10^6$  counts depending on sample concentration. That ACF was  
summed to the running ACF, only when the fitted single exponential  
baseline agreed with the average counts number within 0.3 %. This  
process was stopped when the running ACF reached  $10^7$  counts. At least  
5 intensity ACF were collected and then fitted to a second order cumulant  
30 function of equation 1 (Koppel., *J. Chem. Phys.*, 57:4814-4820;1972),

$$G(t_i) \leftarrow bl \left\{ 1 + sn \left| \exp \left( -\Gamma_1 t_i + \frac{1}{2} \Gamma_2 t_i^2 \right) \right|^2 \right\} \quad (1)$$

where  $G(t_i)$  is the ACF,  $t$  is the time,  $bl$  is the baseline,  $sn$  is a parameter related to the signal to noise ratio, and  $\Gamma_1$  and  $\Gamma_2$  are the first and the second cumulant, respectively.

- 5           The average radius  $R$  is obtained from the first cumulant  $\Gamma_1$  by equation 2,

$$R = \frac{k_B T q^2}{6\pi\eta\Gamma_1} \quad \text{where} \quad q^2 = \frac{4\pi n}{\lambda_0} \sin\left(\frac{\theta}{2}\right) \quad (2)$$

- where  $k_B$  is the Boltzmann constant,  $n$  is the index of refraction of the solution (which depends slightly on buffer and protein concentration),  $q$  is the scattering vector,  $\theta$  is the scattering angle, and  $\eta$  is the solvent viscosity at the experimental temperature  $T$  ( $^{\circ}\text{K}$ ). The latter has been checked within the accuracy of  $0.1^{\circ}\text{C}$  with a thermocouple positioned close to the scattering cell, and it remained within  $\pm 2^{\circ}\text{deg. C}$  of  $24^{\circ}\text{C}$  for the duration of the experiment.  $\lambda_0$  is the wavelength of incident light.

- 15           The ratio of the first two cumulants gives the sample polydispersity ( $Q$ ) according to equation 3:

$$Q = \Gamma_2 / \Gamma_1^2 \quad (3)$$

## B. Results

- 20           Table I reports the radius of HbA as compared to the radius of Hb Prisca at time zero and at increasing polymerization time. The same values were derived from a non linear single exponential fitting procedure of the actual data and from the cumulant analysis of ACF (Eq 2). The number of tetrameric hemoglobins polymerized,  $p$ , was calculated from the

cubic power the ratio of the radius of the polymer over that of tetrameric HbA, assuming that these proteins have a spherical shape at the protein concentration of 1-2 mg/ml. Alternatively this calculation was repeated, considering that at the concentration of the measurements the HbA solution  
 5 contained 10% dimers, i.e. the tetramer radius is derived by equation 4, with  $\alpha=0.1$ :

$$R_T = R_{HbA} \frac{2(1-\alpha) + \alpha\sqrt[3]{2}}{2-\alpha} \quad (4)$$

$P_{50}$  in Table I represents the oxygen affinity at 50% oxygen saturation and  $n$  the cooperativity of the system.

10 **Table I: Biophysical Parameters of HbA and Hb Prisca**

Protein	Time (days)	R (nm)	p	p*	Q	$P_{50}$ (mmHg)	n
HbA	-	3.19±0.01	-	-	0.02±0.02	4.0	2.9
Hb Prisca	0 <sup>#</sup>	3.33±0.05	1.14 ± .05	1.09 ± .05		3.9	2.4
Hb Prisca	6	5.61±0.01	5.44 ± .06	5.22 ± .06		4.7	2.5
15 Hb Prisca	45	6.00±0.05	6.65 ± .18	6.39 ± .17		4.2	2.4
Hb Prisca	90	6.21±0.02	7.38 ± .10	7.08 ± .09		4.0	2.5
Hb Prisca	110	6.20±0.01	7.34 ± .08	7.05 ± .07	0.07±0.02	4.4	2.4

\*Number of tetrameric molecules polymerized, assuming the presence of 10%  $\alpha\beta$  dimers in HbA

<sup>#</sup> These measurements were carried out in the presence of 1mM DTT

20 At zero time the hydrodynamic radius of HbA and Hb Prisca was the same. After removal of DTT, the hydrodynamic radius rapidly increased to indicate the polymerization of five tetrameric molecules. The highest degree of polymerization was attained after 45 to 90 days consistent with the decreased motion of the polymeric molecules.



The non-linear fit cumulant analysis (Eq. 1) of HbA and of Hb Prisca at 110 days polymerization time is shown in Figs. 1 and 2. The shift of Hb Prisca toward the right indicates a slower decay rate, consistent with a larger radius of the polymer. The residual for the analysis of the polymerized Hb Prisca in comparison to unpolymerized HbA indicates the presence of a homogeneous population in the polymerized mutant hemoglobin.

The linear cumulant analysis of HbA and of Hb Prisca at 110 days polymerization time is compared in Fig. 3. For each protein the results from five different measurements are reported; they are very similar. The different slope of HbA and Hb Prisca again reflects the different size of the two samples. The two samples have a remarkable similar linearity, consistent with the presence of a highly homogeneous population.

The values of polydispersity  $Q$ , (Table I) representing the deviation from a perfect homogeneous sample ( $Q=0$ ), is  $0.02 \pm 0.02$  for HbA, indicating the perfect monodispersity of this sample and  $0.07 \pm 0.02$  for Hb Prisca at 110 days polymerization time. The small polydispersity calculated for Hb Prisca indicates that the amount of non homogeneous population present in the polymerized sample, is very small.

20

### **Example 5**

#### **Functional Characterization of Hb Prisca**

##### **A. Methods**

Aliquots were taken at time intervals during polymerization for oxygen binding measurements. Prior to measurements, CO was removed photolysis under a stream of oxygen. Measurements were performed using the thin layer dilution technique of Dolman and Gill (*Anal. Biochem.* 87:127-134, 1978) with an AVIV 14DS spectrophotometer. In this technique the oxyhemoglobin is deoxygenated by stepwise dilution of the equilibrating gas with constant volumes of  $N_2$ . Formation of methemoglobin as judged by spectral deconvolution of the initial and final spectra was less

than 5%. Protein concentration was 1.5-2.0 mM in heme, in 50 mM HEPES +100mM NaCl+1mMEDTA buffer at pH 7.4 at 24°C. The experimental data were fitted to the Adair equation using an iterative procedure incorporating the Marquardt algorithm,

$$Y = \frac{\beta_1 p(O_2) + 2\beta_2 p(O_2)^2 + 3\beta_3 p(O_2)^3 + 4\beta_4 p(O_2)^4}{4[1 + \beta_1 p(O_2) + \beta_2 p(O_2)^2 + \beta_3 p(O_2)^3 + \beta_4 p(O_2)^4]} \quad (5)$$

- 5 where Y is the fractional saturation with O<sub>2</sub>, P(O)<sub>2</sub> is the partial pressure of oxygen in millimeters of mercury and β<sub>is</sub> are the overall Adair constants related to the intrinsic statistical affinity constant K<sub>i</sub> of the subsequent steps of oxygenation by β<sub>i</sub> = ∏<sub>j=1</sub><sup>i</sup> K<sub>j</sub>. The value of the median ligand activity P<sub>m</sub>, was determined using the relation P<sub>m</sub> = β<sub>4</sub><sup>-0.25</sup> (Wyman, J and Gill, S. J.  
10 *Binding and Linkage: Functional Chemistry of Biological Molecules*. University Science Books. Mill Valley, CA.)

## B. Results

The functional parameters of Hb Prisca calculated at increasing polymerization time are listed in Table I, above. The functionality at pH 7.4  
15 was not modified by the extent of polymerization. This indicates that the intermolecular crosslinking at the two external β9Cys and the packing of the seven hemoglobin molecules do not introduce conformational constraints to either the tertiary or quaternary structure which affect the functionality at physiological pH.

20 Fig. 4 contains the oxygen binding curves of natural HbA and of Hb Prisca at 110 days polymerization time are shown. The two hemoglobins have a similar oxygen affinity, but the cooperativity of Hb Prisca, although good (n=2.4), is decreased with respect to HbA (n=2.9). See Fig. 5. A similar decrease in cooperativity is observed in the wild type of the  
25 recombinant hemoglobins prepared with the same expression system (Fronticelli *et al.*, *J. Biol. Chem.* 270:30588-30592 (1995), Sanna *et al.*, *J. Biol. Chem.* 272: 3478-3486 (1996)), and in the β cysteine single and

double mutants, in similar experimental conditions of buffer and Cl<sup>-</sup> concentration (Dolman and Gill, *supra*). Thus, the observed decrease in cooperativity observed in Hb Prisca is intrinsic to the system and should not be attributed either to the mutations or to the polymerization phenomenon.

5 This is at variance with glutaraldehyde polymerization of HbA which gives a highly heterogeneous product with a largely reduced cooperativity ( $n=1.3-1.5$ ) (Doyle *et al.*, *J. Biol. Chem.* 274:2583-2591, 1999; MacDonald & Pepper, *Methods in Enzymology* 231:287, 1994).

All references discussed herein are incorporated by reference. One

10 skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The present invention may be embodied in other specific forms without departing from the spirit or essential attributes thereof and, accordingly, reference should be made to

15 the appended claims, rather than to the foregoing specification, as indicating the scope of the invention.

**CLAIMS**

1. A human  $\beta$ -globin mutant polypeptide comprising the amino acid sequence of the normal human  $\beta$ -globin modified by (i) the substitution or deletion of Cys at positions 93 and 112, and (ii) the substitution of a Cys for a non-Cys amino acid at one other position in the polypeptide.  
5
2. A mutant polypeptide according to claim 1 wherein Cys is substituted for one of the amino acids at the following positions:
  - (a) Ser at position 9;
  - (b) Asn at position 80; or
  - 10 (c) Lys at position 17.
3. A mutant polypeptide according to claim 1 wherein Cys is substituted for Ser at position 9.
4. A mutant polypeptide according to claim 3 having the amino acid sequence SEQ ID NO:4.
- 15 5. A nucleic acid sequence encoding a mutant polypeptide according to claim 4.
6. A nucleic acid according to claim 4 which comprises the nucleotide sequence SEQ ID NO:2.
7. A vector comprising a promoter operably linked to a nucleic acid sequence according to claim 6, capable of directing the expression of a mutant human  $\beta$ -globin polypeptide.  
20
8. A host cell transformed with a vector according to claim 7.

9. A method for producing a human  $\beta$ -globin mutant polypeptide comprising:

5 growing a culture of transformed host cells of claim 8 under conditions conducive to the expression of said polypeptide by said host cells.

10. A modified human hemoglobin comprising a  $\beta$ -globin polypeptide according to claim 1.

11. A modified human hemoglobin comprising a  $\beta$ -globin polypeptide according to claim 2.

10 12. A modified human hemoglobin comprising a  $\beta$ -globin polypeptide according to claim 3.

13. A modified human hemoglobin comprising a  $\beta$ -globin polypeptide according to claim 4.

15 14. A modified human hemoglobin according to any of claims 10-13 comprising a mutant human  $\alpha$ -globin polypeptide comprising the amino acid sequence of normal human  $\alpha$ -globin modified by the substitution or deletion of Cys at position 104.

20 15. A modified human hemoglobin according to claim 13 comprising a mutant human  $\alpha$ -globin polypeptide comprising the amino acid sequence of normal human  $\alpha$ -globin modified by the substitution or deletion of Cys at position 104, and further modified by either the substitution of Cys for Ala at position 71, or the substitution of Cys for Ala at position 53.

16. A modified human hemoglobin comprising:  
a mutant human  $\alpha$ -globin polypeptide comprising the amino acid sequence of normal human  $\alpha$ -globin modified by the substitution of Cys at position 104 by a non-Cys amino acid;  
5 a mutant human  $\beta$ -globin polypeptide comprising the amino acid sequence of normal human  $\beta$ -globin modified by the substitution of Cys at positions 93 and 112 by non-Cys amino acids; and  
said modified hemoglobin further characterized by the substitution of Cys for the native sequence amino acid at one of the following positions:  
10  $\beta$ -globin position 9;  
 $\beta$ -globin position 17;  
 $\beta$ -globin position 80;  
 $\alpha$ -globin position 71; or  
 $\alpha$ -globin position 53.
- 15 17. A polymeric hemoglobin comprising a modified human hemoglobin according to claim 10, 11, 12, 13 or 16, wherein adjacent hemoglobins are covalently bonded to each other by one or more disulfide bridges formed by cysteine amino acid residues.
- 20 18. A polymeric hemoglobin according to claim 17 wherein the modified hemoglobin is characterized by the substitution of Cys for the native sequence amino acid at  $\beta$ -globin position 9, said polymeric hemoglobin comprising seven modified hemoglobins.
19. A blood substitute comprising a polymeric hemoglobin according to claim 17.
- 25 20. A blood substitute comprising a polymeric hemoglobin according to claim 18.

21. A method of supplementing the oxygen-carrying capacity of a patient's blood comprising administering to the patient an effective amount of the blood substitute according to claim 19.
22. A method of supplementing the oxygen-carrying capacity of a patient's blood comprising administering to the patient an effective amount of the blood substitute according to claim 20.
23. A mutant human  $\alpha$ -globin polypeptide comprising the amino acid sequence of normal human  $\alpha$ -globin modified by the substitution or deletion of Cys at position 104.
24. A mutant human  $\alpha$ -globin polypeptide comprising the amino acid sequence of normal human  $\alpha$ -globin modified by the substitution or deletion of Cys at position 104, and further modified by either the substitution of Cys for Ala at position 71, or the substitution of Cys for Ala at position 53.

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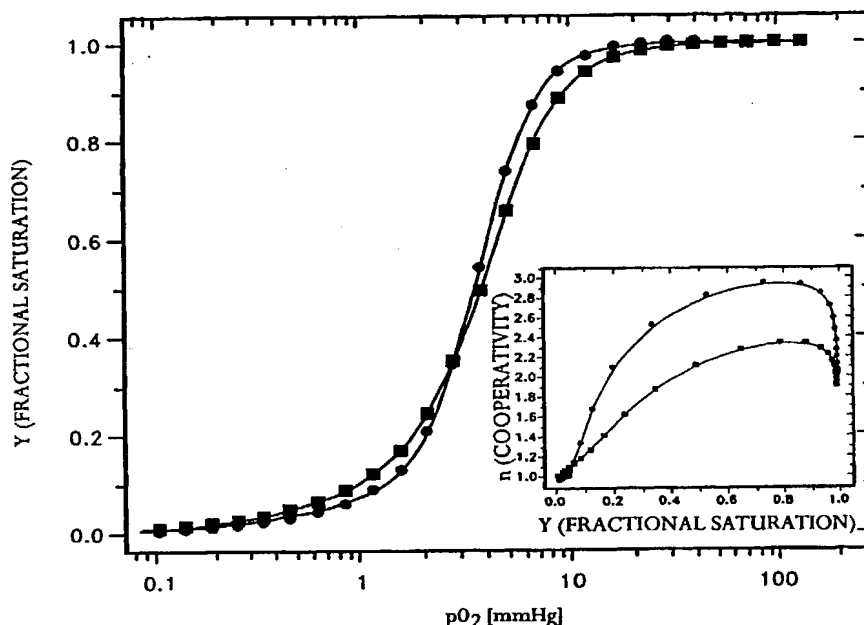
(74) Agent: MONACO, Daniel, A.; Seidel, Gonda, Lavorgna & Monaco, P.C., Suite 1800, Two Penn Center Plaza, Philadelphia, PA 19102 (US).

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(57) Abstract

Human  $\beta$ -globin mutant polypeptides comprise the amino acid sequence of normal human  $\beta$ -globin modified by (i) the substitution or deletion of Cys at positions (93) and (112), and (ii) the substitution of a Cys for a non-Cys amino acid at one other position in the polypeptide, preferably Ser at position (9). Modified human hemoglobins containing this or other  $\beta$ -globin mutations, and/or an  $\alpha$ -globin mutation, are provided, as well as polymers of such modified hemoglobins. Nucleic acids, vectors and transformed host cells for producing the  $\beta$ -globin mutants are provided.



1/3

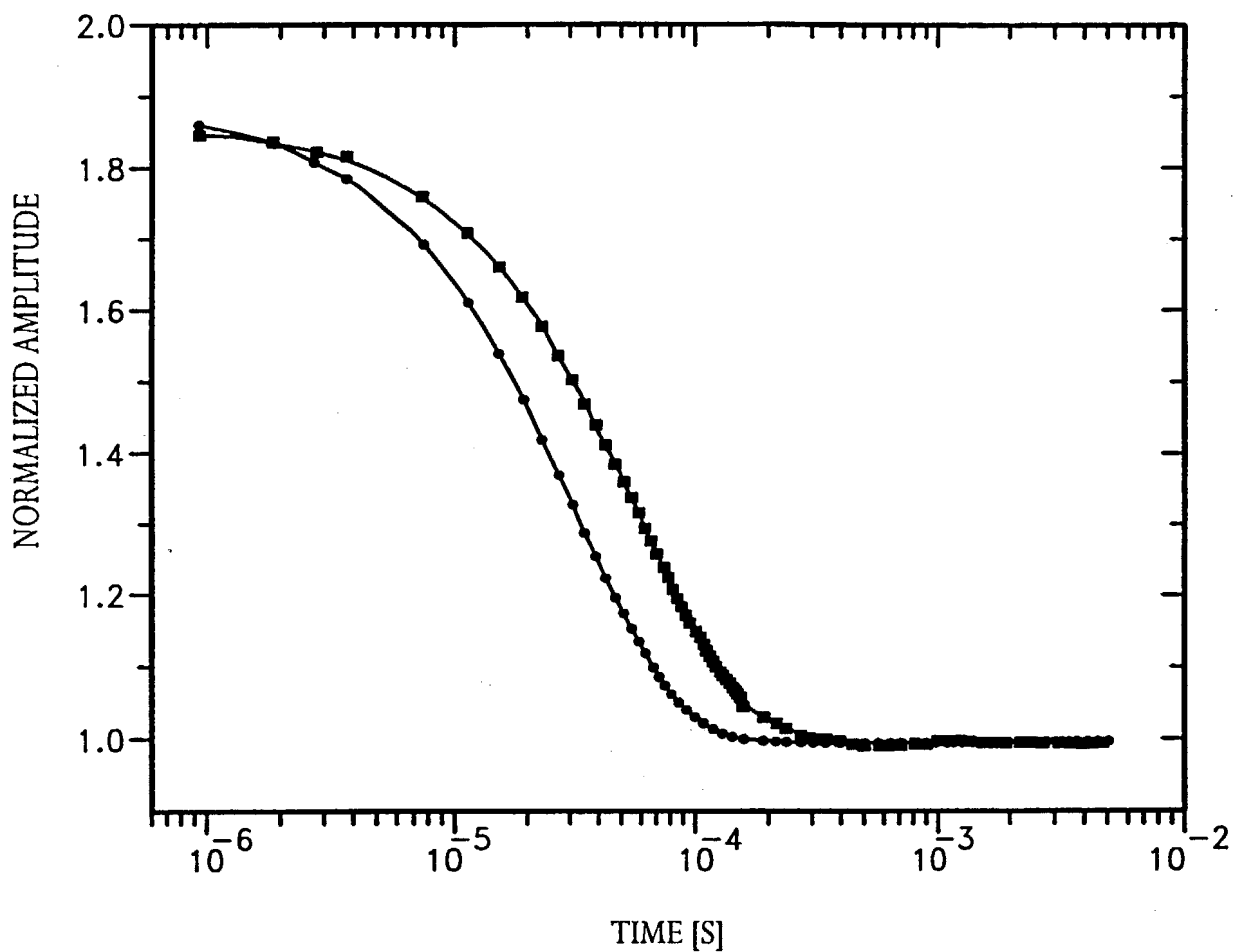


FIG. 1

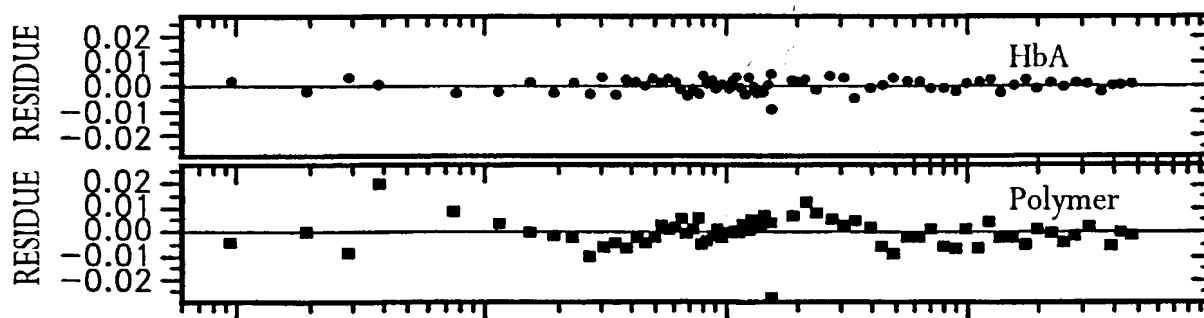


FIG. 2

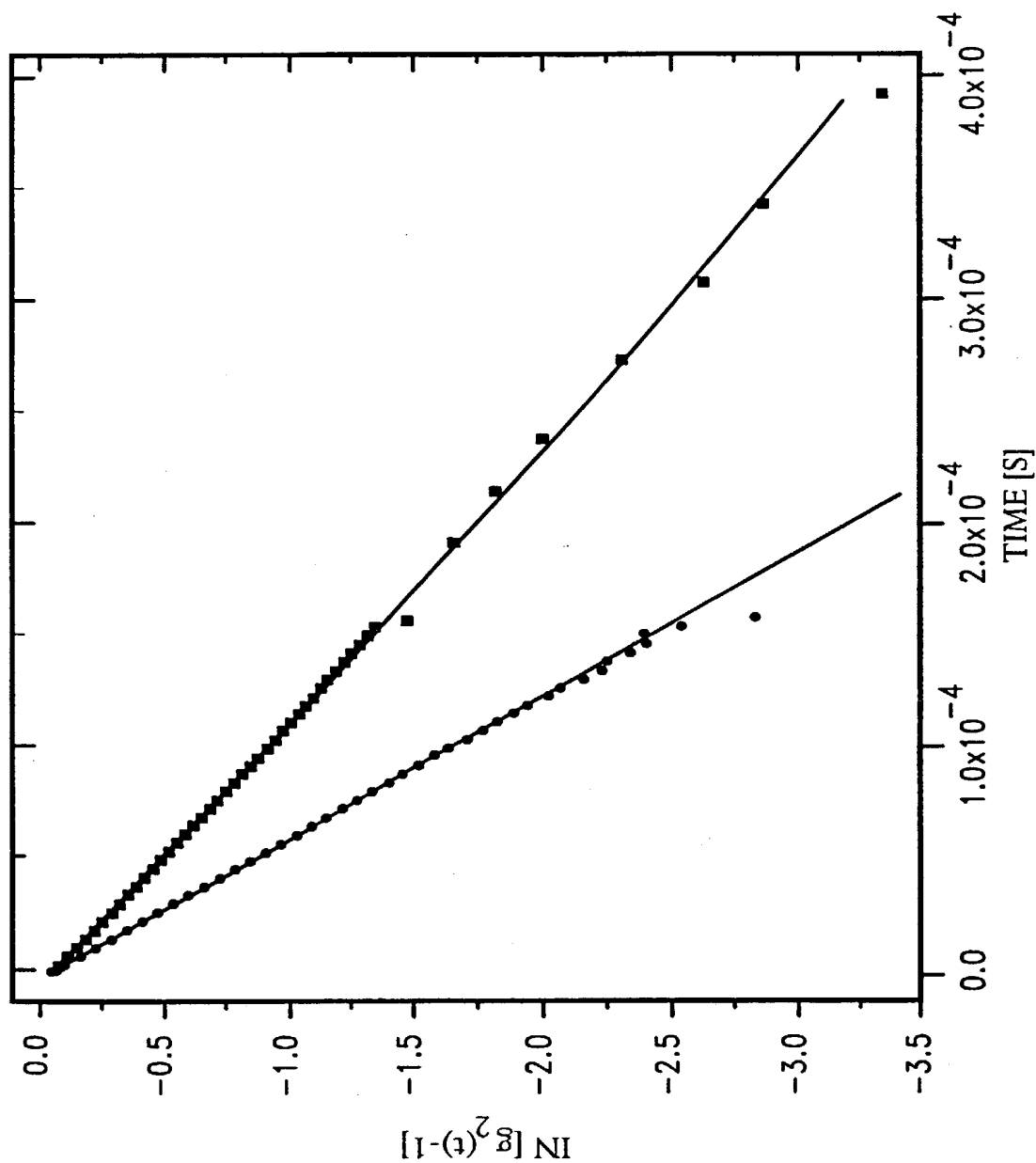


FIG. 3

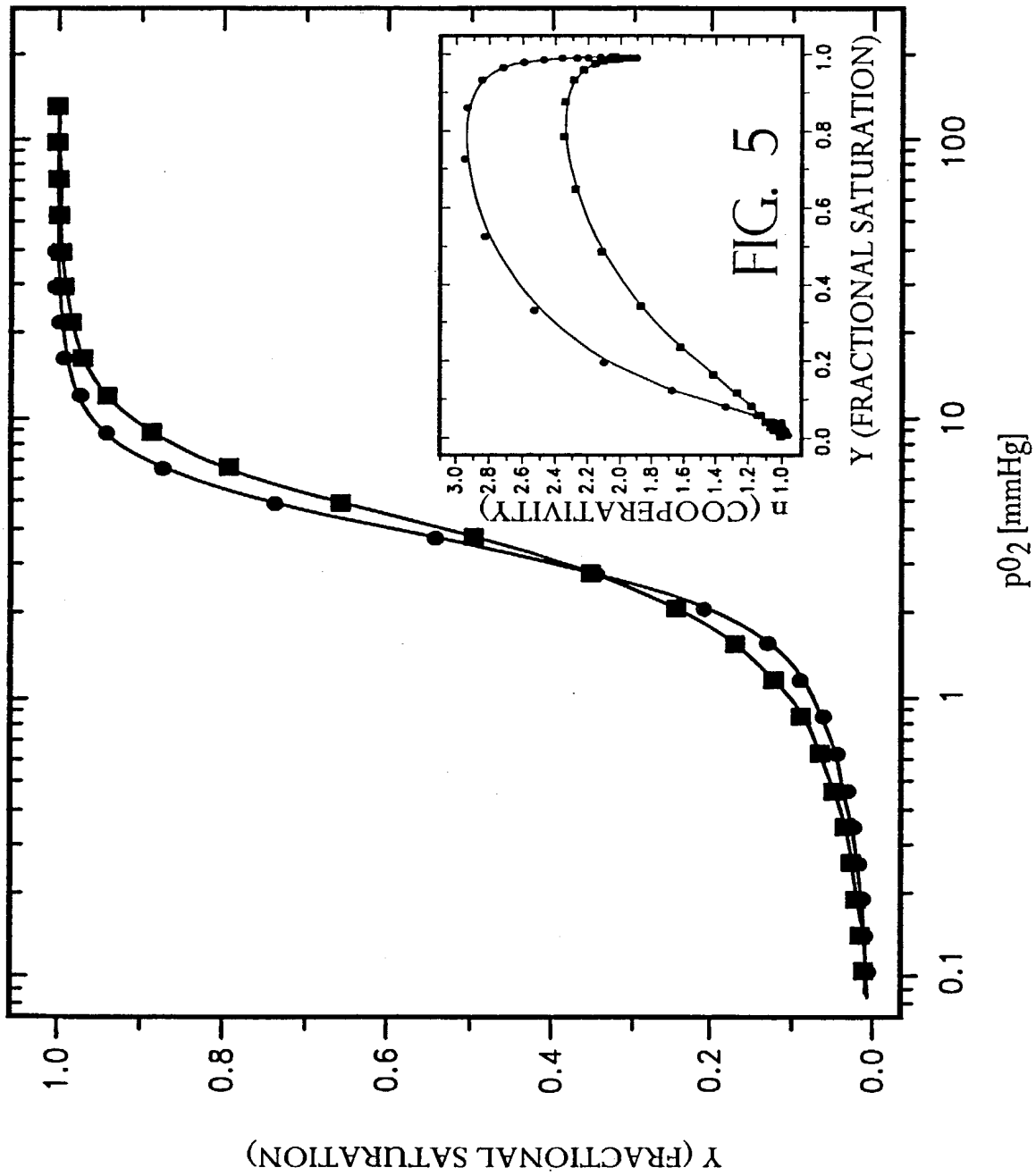


FIG. 4

**PATENT**

**ATTORNEY DOCKET #:**6056-279

**DECLARATION AND POWER OF ATTORNEY**

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are stated below next to my name.

I believe I am the original, first, and sole inventor (if only one name is listed below) or an original, first, and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

**POLYMERIC HEMOGLOBIN MUTANTS**

the specification of which is attached hereto unless the following box is checked

☒ was filed on 30 September 1999 as PCT Application No. PCT/US99/22756.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with 37 CFR §1.56.

I hereby claim foreign priority benefits under 35 U.S.C. §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT international application which designated at least one country other than the United States, listed below and have also identified below any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed:

**PRIOR FOREIGN/PCT APPLICATION(S)**

<b>COUNTRY/OFFICE</b>	<b>APPLICATION NO.</b>	<b>DATE OF FILING</b>	<b>PRIORITY CLAIMED</b>
			<input type="checkbox"/> YES <input type="checkbox"/> NO

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below.

<b>PROVISIONAL APPLICATION NUMBER</b>	<b>DATE OF FILING</b>
60/102,640	October 1, 1998

I hereby claim the benefit under 35 U.S.C. §120 of any United States application(s) or §365(c) of any PCT International application(s) designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of 35 U.S.C. §112, I acknowledge the duty to disclose material information as defined in 37 CFR §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

**PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS  
DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. §120**

STATUS (CHECK ONE)

APPLICATION SERIAL NO.	DATE OF FILING	PATENTED	PENDING	ABANDONED
none		<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

And I hereby appoint Arthur H. Seidel, Registration No. 15,979; Gregory J. Lavorgna, Registration No. 30,469; Daniel A. Monaco, Registration No. 30,480; Thomas J. Durling, Registration No. 31,349; and John J. Marshall, Registration No. 29,671, my attorneys or agents with full power of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.

Address all correspondence to Seidel, Gonda, Lavorgna & Monaco, P.C., Suite 1800, Two Penn Center Plaza, Philadelphia, Pennsylvania 19102. Address all telephone calls to Daniel A. Monaco (215)568-8383 (telefax: 215-568-5549).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

**FULL NAME OF SOLE OR FIRST INVENTOR**

Clara

(GIVEN NAME)

Fronticelli

(MIDDLE INITIAL OR NAME)

(FAMILY OR LAST NAME)

**Inventor's signature:**

Clara Fronticelli

**Date:**

2-22-2000

**Country of Citizenship:**

United States

**Residence:**

Timonium

Maryland

(City)

(State or Foreign Country)

**Post Office Address:**

11514 Pebble Creek, Timonium, Maryland 21093

# SEQUENCE LISTING

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 35 40 45  
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 50 55 60  
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			20					25					30		
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		35					40					45			
Ser	His	Gly	Ser	Ala	Gln	Val	Lys	Gly	His	Gly	Lys	Lys	Val	Ala	Asp
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